CHARACTERIZATION OF *BOTRYOCOCCUS SP.* AND IDENTIFICATION OF CARBOHYDRATES METABOLIC RELATED ENZYMES TOWARD LIPID PRODUCTION

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I dedicate this work with the most profound sense of gratitude to Almighty Allah for giving me Strength and Inspiration in making my dreams come true.

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ABSTRACT

The advancement of microalgal biofuels is faced with challenges among which is low strain performance in lipid production. Alternatively, the carbohydrate production in microalgal cells could be manipulated to increase the lipid content thereby maximizing the overall biofuel production. This work is aimed at determining metabolic pathway and enzymes involved in carbohydrate metabolism of *Botryococcus sp.* The *B. sp.* from Taman Negara Johor Endau Rompin was isolated and identified. Some relevant intracellular metabolites were extracted and quantified through HPLC and GC-MS analysis while extracellular metabolites excreted into the Bold basal medium were also analysed and identified through GC-MS. Enzymes involved in carbohydrate metabolism leading to lipid production in *B. sp.* under natural conditions were also identified through one-dimensional gel electrophoresis followed by proteomic mass spectrometry (LC-MS) and database searching. Finally, the carbohydrate to lipid metabolic pathway of *B. sp.* cultivated under natural conditions was determined. The total carbohydrate content was found to be 23 % per milligram biomass dry weight with monomeric sugars galactose, glucose, mannose, and arabinose. Total protein estimated for the microalgae *B. sp.* is 16.22 % and the lipid content was found to be 60.69 %. The extracellular metabolites constitute majorly cyclohydrocarbons, nitrogenated hydrocarbons, siloxanes, phenols and phenol derivatives. A glycolytic enzyme 'Enolase 'which can generate phosphoenolpyruvate (PEP) and then convert it into pyruvate was identified in this study. Enolase which helps in high lipid metabolism was found in the cytoplasm and was used to construct the alternative pathway. Enolase was found to export fixed carbon (3PGA) to the cytoplasm, hence providing a shorter route to lipid production than the normal process via the plastid leading to the production of more lipid in *Botryococcus*.

ABSTRAK

Kemajuan biofuels biojisim berhadapan dengan cabaran di mana prestasi strain adalah rendah dalam pengeluaran lipid. Selain itu, pengeluaran karbohidrat dalam sel-sel mikroalga boleh dimanipulasi untuk meningkatkan kandungan lipid, seterusnya memaksimumkan pengeluaran biofuel secara menyeluruh. Kajian ini bertujuan untuk menentukan laluan metabolik dan enzim yang terlibat dalam metabolisma karbohidrat *Botrycoccus sp*. *B. sp.* dari Taman Negara Johor Endau Rompin diasing dan dikenalpasti. Sesetengah metabolit intraselular yang berkaitan telah diekstrak dan dikira melalui analisis HPLC dan GC-MS manakala metabolit ekstraselular yang dikumuhkan ke dalam medium basal Bold juga dianalisis dan dikenalpasti melalui GC-MS. Enzim yang terlibat dalam metabolisma karbohidrat yang membawa kepada pengeluaran lipid dalam *B. sp.* dibawah keadaan semulajadi juga dikenalpasti melalui elektroforesis gel satu dimensi diikuti dengan spektrometri jisim proteomik (LC-MS) dan pencarian pangkalan data. Akhirnya, laluan metabolik karbohidrat ke lipid *B. sp.* terkultur di bawah keadaan semulajadi telah ditentukan. Jumlah kandungan karbohidrat didapati 23 % per miligram berat kering biomas dengan gula monomerik galaktosa, glukosa, mannosa, dan arabinosa. Jumlah protein yang dianggarkan untuk *B. sp.* mikroalga adalah 16.22 % dan kandungan lipid didapati 60.69 %. Metabolit ekstraselular merupakan sebahagian besar daripada siklohidrokarbon, hidrokarbon nitrogenasi, siloxan, fenol dan terbitan fenol. Enzim glikolitik 'Enolase' yang boleh menghasilkan fosfoenolpyruvat (PEP) dan kemudian mengubahnya menjadi piruvat telah dikenalpasti dalam kajian ini. Enolase yang membantu dalam metabolisma lipid tinggi didapati di sitoplasma dan digunakan untuk membina laluan alternatif. Enolase ditemui untuk mengeksport karbon tetap (3PGA) ke sitoplasma, dengan itu memberikan laluan yang lebih pendek ke pengeluaran lipid daripada proses biasa melalui plastid yang membawa kepada pengeluaran lebih banyak lipid dalam *Botryococcus.*

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LIST OF ABBREVIATIONS

ATP	-	Adenosine Triphosphate
APS	-	Ammonium Persulfate
BBC	-	Bold Basal Medium
BSA	-	Bovine Serum Albumin
CC	-	Calvin Cycle
CO_2	-	Carbon IV Oxide
CO_3^{2-}	-	Carbonate
$CaC1_2$	-	Calcium Chloride
DDT	-	Dithiothreitol
DDH_2O	-	Double Distilled Water
DHA	-	Docosahearnoi Acid
DNA	-	Dioxyribonucleic Acid
EDX	-	Energy Dispensive X- Ray
ER	-	Endoplasmic Reticulum
EPA	-	Eicosapentaenoicacid
ESP	-	ExoPolysaccharide
GHG	-	Green House Gases
$H_2PO_4^-$	-	hydrogen phosphate ion
HPO_4^{2-}	-	hydrogen phosphate
HPLC	-	High Performance Liquid Chromatography
IAA	-	Iodoaetamide
LC- MS	-	Liquid Chromatography Mass Spectrometer
MG	-	Magnesium
NaCl	-	Sodium Chloride
NADP+	-	Nicotinamide Adenine dinucleotide Phosphate
$NaNO_3$	-	Sodium Nitrate
NREL	-	Renewable Energy Laboratory

NO_3	-	Nitrate
O_2	-	Oxygen
P	-	Phosphorus
PRB	-	Photo Bioreactor
PGA	-	Phosphoric Acid
PUFA	-	Polyunsaturated Fatty Acid
PAGE	-	Polyacrylamide Gel Electrophoresis
RUBISCO	-	Ribulose 1, 5 - Biphosphate carboxylase
RNA	-	Ribonucleic Acid
SI	-	Siloxane
SDS	-	Sodium Chloride Sulphate
TAG	-	Triacygly Ceride or Triglycerides
TEMED	-	Tetrametylethylenediamine
UTHM	-	Universiti Tun Hussein Onn Malaysia

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CHAPTER 1

INTRODUCTION

1.1 Background

Currently, fossil fuels provide about 80 % of global energy demand (Petroleum, 2012). Issues such as decreasing reserves, inadequate supply, and unavoidably growing market, the world's cheapest source of energy is still fossil fuels. However, the prices of fossil fuels have increased as demand has exceeded supply. Therefore, many nations have started to ponder the problem of energy safety, mostly as fossil fuels are their only energy source. Consequently, widespread exploitation of fossil fuels has caused organic greenhouse gases (GHG) release, in which more than 60% is carbon dioxide that is mostly responsible for global climate change. Furthermore, the environmental impact has an enormous effect on the carbon cycle (carbon balance), which is related to the combustion of fossil fuels. Besides, exhaustion of different existing biomass without appropriate compensation resulted in colossal biomass scarcity, developing environmental problems like deforestation and the destruction of biodiversity.

Improving the rate of reduction of fossil fuels and reducing GHG emissions needs sufficient renewable clean energy to be generated for use. Small to medium-term renewable and environmentally friendly alternatives for fossil fuels have lately attracted the serious attention of sciences worldwide (Ghaffour *et al.*, 2015).

Among several possible options, at present is biofuels. This is one of the utmost interest and are likely to show a significant role in global energy substructure. Biofuels are fuels that comprise energy from geologically current carbon fixation by living organisms. Biofuels produced from biomasses are usually from plants or materials originating from plants or biomasses from algae and microalgae, which are biodegradable, non-toxic and renewable (Ioelovich, 2013, 2015; Song *et al.*, 2008). They use solar energy, together with carbon dioxide, to convert and store the biomass in the forms of carbohydrates, lipids, and proteins through the process of photosynthesis (Liu *et al.*, 2015).

Microalgae is a unicellular organism that executes higher photosynthetic effectiveness and growth potential compared with terrestrial plants (Katiyar *et al.*, 2017). Since it has high photosynthetic efficiency, the mass cultivation can reduce the carbon dioxide emission efficiently from the atmosphere, hence, decreasing the effect of global warming (Surriya *et al.*, 2015). According to Milano *et al.* (2016), microalgae have a high growth rate and can produce up to 70 % of lipid content inside their cells depend on the type of species. They also reported that microalgae can survive under the severe condition and can grow in a small cultivation land area. Its cultivation does not compete with land plants with the growth of crops for food and biofuel and never require rigorous treatment methods in comparison with lignocellulose-enriched biomass. Furthermore, microalgae can grow on nonagricultural soil using wastewater instead of drinking water (Gani *et al.*, 2016). They accumulate carbohydrates and lipids, which are the most abundant constituents of microalgal cells, in addition to other valuable chemicals, such as carotenoids and proteins (Jeon *et al.*, 2017).

The existence of the microalga *Botryococcus* sp. in America, Africa, Asia and Europe has confirmed its wide distribution (Chandra, 1964; Chandrappa *et al.*, 2010; Metzger *et al.*, 1985; Okada *et al.*, 2000; Wolf *et al.*, 1985). Furthermore, these geographical regions belong to different climatic zones like continental, temperate, tropical and alpine, hence an indication of its ability to grow in varied climatic conditions (Tyson, 2012). The ability of microalgae to survive in diverse and extreme conditions is reflected in its tremendous diversity and also the unusual pattern of cellular lipids obtained from these microalgae (Sato *et al.*, 2000). Furthermore, some of these microalgae can also modify lipid metabolism efficiently to counter adverse changes in environmental conditions (Guschina & Harwood, 2006; Thompson Jr, 1996). The most prominent characteristic of *Botryococcus* is its ability to synthesize and accumulate very high levels of lipids. These lipid substances include numerous hydro-carbons, that is, highly reduced compounds comprising of only carbon and hydrogen as elements (Brown *et al.*, 1969). *Botryococcus* being a photosynthetic organism, has been reported to reduce CO_2 emissions by 1.5×10^5 tons/yr./8.4×10^3

ha (Sawayama *et al.*, 1999) thereby offering an eco-friendly process for production of lipid and other bioactive compounds along with its carbon dioxide mitigation credits.

Additionally, *Botryococcus* spp. are well known to have a remarkable capability to synthesise industrially interesting chemicals intracellular and also excrete them into the extracellular matrix. Although *Chlorella* and *Dunaliella* are both highly efficient microalgae with regards to growth-to-lipid ratio, *Botryococcus* has outclassed them both. *Botryococcus* can produce a large number of hydrocarbons as compared to its biomass (Chisti, 2007a; Cohen, 2014). The production level of oil content in microalga *Chlorella* is up to 50% lipids, while *Botryococcus* produces the highest oil content of approximately 80% (Powell & Hill, 2009).

Due to its potential to produce large amounts of lipids and hydrocarbons, exploration of the genus *Botryococcus* is still ongoing for newer strains and species and to date, more than 60 *Botryococcus* strains have been cultivated in laboratories (For a non-exhaustive list, see Metzger and Largeau, 1999) and the exploration is still on. Most *Botryococcus* strains cultivated in laboratories and wild samples collected from lakes worldwide are analyzed for their lipid production (Metzger and Largeau, 1999) and are reported to produce 25 - 75% of lipids on their dry weight (Metzger and Largeau, 1999, 2005; Dayananda et al., 2007a; Ranga Rao et al., 2007; Hai-Linh et al., 2009; Ela and Anastasios, 2010; Chiara et al., 2010).

However, there are no reports available regarding why and what is responsible for the high lipid content in *B. sp.* in comparison to other microalgae. In view of its importance, the present study aims to find out the cause from an indigenous species of *Botryococcus* isolated in TJNER.

To enhance the economic feasibility of mass production of micro-algal biofuels, several efforts are still being made to increase strain performance (Xin *et al.*, 2010), cultivation methods (Ketheesan & Nirmalakhandan, 2012; Zhang & Hu, 2012) and improving technologies involved in biomass harvesting and extraction (Halim *et al.*, 2012; Hanotu *et al.*, 2012).

1.2 Statement of the problem

The rising interest surrounding the continued use of fossil fuels and rapid reduction of fossil fuel reserves, global climate change, increasing crude oil price and environmental degradation has enforced governments, policymakers, scientists, and researchers worldwide to find alternative renewable energy sources like biofuels (Alam *et al.*, 2015; Dragone *et al.*, 2010). Microalgae have emerged as a promising feedstock for the production of biofuels leading to explorations for a suitable microalgal strain for biotechnological exploitation with a higher lipid content than what is obtainable now.

Botryococcus spp. have been reported to be richer in lipid content (Huang et al. 2013) than other microalgae with also a significant high lipid content for example, C*hlorella vulgaris* and *Dunaliella* (Griffiths and Harrison 2009; Keffer and Kleinheinz 2002).This begs the question of why or what is the cause of this disparity in lipid content between *Botryococcus* and other microalgal species.

Furthermore, according to Cakmak *et al. (*2012), Hu *et al.* (2008), Li *et al. (*2011) and Siaut *et al. (*2011), photosynthetic carbon partitioning switch towards the production of energy-rich storage compounds like starch and lipid, in which it can be converted into biofuels is possible. In other words, the carbohydrate present in cells can be converted to lipids by manipulating the carbohydrate pathway to increase the lipid content, hence maximising the overall biofuel production.

Therefore, understanding the metabolic pathways and processes involved in the generation of these organic macromolecules is essential to improve biofuels production. However, studies on carbohydrate metabolic pathway and enzymes discovery leading to the production of lipid in *B. sp.* are minimal. Hence the need to study the metabolic pathways and identify the enzymes involved in the metabolism of carbohydrates in *B. sp.* that will lead to more lipid production with the hope of improving its biofuel productivity. Conversely, microalgae have a strategic feature of rapid alteration of the intracellular energy storage form from starch to lipids, thereby generating more lipids contents. Overall, this is a fundamental study into the basic composition and metabolic biosynthesis of the microalga *Botryococcus* sp.

1.3 Research aim and objectives

This research work aims to determine the metabolic pathway and the enzymes involved in carbohydrate metabolism of *B. sp.* The objectives of this study include:

❖ To identify the intercellular and extracellular metabolites produce from *B. sp.*

cultured under natural conditions.

- To evaluate the enzymes involved in carbohydrate metabolism leading to lipid production in *B. sp.* under natural conditions.
- To construct an alternative carbohydrate to lipid metabolic pathway of *B. sp.* cultivated under natural conditions.

1.4 The importance of the study

Renewable and maintainable energy sources from photosynthetic microalgae have received much attention in recent years. *B. sp.* is capable of converting sunlight and CO_2 into biomass and other valuable products. *B. sp.* was initially collected from Endau Rompin National Park and known as a wild type of microalga. The microalgae are an indigenous species and have not been studied intensively yet. Because of its virtually unique capability to synthesise large quantities of exopolysaccharides and hydrocarbons, this colony-forming green microalga serves as a potential source for industrial applications like biofuels and other valuable chemicals.

However, there is little understanding of the metabolism that will channel polysaccharides to this lipid production. Through studying the intracellular and extracellular metabolites, it enabled us to know the mass balance inside and outside of the microalgae since we understand what it consumes. This study also contributes to giving much understanding on the mechanism of the carbohydrate pathway based on the identified proteins/enzymes as a primary novelty to enhance the potential of biofuel production in future.

This study will also aim to establish an alternative pathway of lipid synthesis which will not be based on the genetic modification but the export of the fixed carbon (3PGA) to the cytoplasm, providing a shorter route to lipid production than the normal process via the plastid. This pathway will prove to be a different route to producing more lipid that was not established before. The alternate pathway will also show the main reason why the *B. sp.* was able to produce more lipids than carbohydrate.

1.5 The scope of the study

This research was carried out to determine and identify the enzymes involved in carbohydrate metabolism leading to lipid production and to construct this pathway subsequently. The study covered the culture *B. sp.* isolated from the rocky pools of Taman Negara Endau Rompin, Johor, Malaysia. The microalgae were mass cultured in Bold basal medium (BBM) at an ambient temperature of 25 °C to 30 °C pending on the time of the day within the lumination of 30-60 W/M^2 from sunlight, harvested and freeze-dried. Proximate analysis to determine the percentage of carbohydrates, lipid and proteins contents was carried out. Additionally, the monomeric composition of sugars, fatty acids, characterisation of extracted proteins to identify enzymes and the metabolites produced by the microalga were determined. Finally, the carbohydrate pathway was constructed based on the information gathered from the previous analysis.

CHAPTER 2

LITERATURE REVIEW

2.1 Microalgae, *Botryococcus* sp.

Microalgae are considered to be the oldest living organisms on the planet (Groombridge *et al.*, 2002; Richmond, 2008). They are the primary producers and can fix nearly 50 % of the global carbon (77 %) with the production of oxygen, O2 (Metz *et al.*, 2007). Rawat *et al.* (2013) reported that more than 50,000 microalgae species existing in the world but estimated only 30,000 identified. Depending on the species of microalgae, their sizes ranged from micrometres to millimetres. The term microalgae have no formal taxonomic standing and are defined as thallophytes lacking roots, stems, and leaves with chlorophyll as their primary photosynthetic pigment (Brennan & Owende, 2010; Kumar *et al.*, 2013; Saifullah *et al.*, 2014). They are prokaryotic organisms with simple cellular structure and a large surface to volume body ratio, which offers them the capacity to uptake a lot of nutrients (Fogg, 2012). They grow very fast and live in different environmental conditions such as the aquatic and terrestrial ecosystems (Mata *et al.*, 2010; Saifullah *et al.*, 2014).

The green algae are one of the largest algae groups regarding species number, biological distribution, and morphological diversity. The eukaryotic algal species, the green algae are found in an enormous variety of habitats (Wehr *et al.*, 2015). They occur in lakes, freshwater, streams, and ponds also on and in the soil, rocks, snow, ice, plants, and animals. Lastly, algae can also be free-living or be in a relationship with other organisms, such as lichens (Sankaran & Thiruneelagandan, 2015). Morphological differentiation is ranging from unicellular strains (free-living

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